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(71) Applicant: **NAKANO VINEGAR CO., LTD.**
2-6, Nakamura-cho
Handa-shi, Aichi-ken 475(JP)

(72) Inventor: **Tamaki, Toshimi**
2-11 Arako-cho, Handa-shi
Aiichi-ken, 475(JP)
Inventor: **Takemura, Hiroshi**
101 Bonuru-Ichibankan, 2-4-3 Shimomae,
Toda-shi, Saitama-ken, 335(JP)
Inventor: **Tayama, Kenji**
2-201 Koupo-Nomura-Handa, 2-17
Horisaki-cho
Handa-shi, Aichi-ken 475(JP)
Inventor: **Fukaya, Masahiro**
1-28 Morioka-Aza-Nigoriike, Higashira-cho
Chita-gun, Aichi-ken 470-21(JP)
Inventor: **Okumura, Hajime**
5-66-14 Yanabe-Higashi-machi, Handa-shi
Aichi-ken, 475(JP)
Inventor: **kawamura, Yoshiya**
132 Furawatar, Kocjino-cho,
Kounan-shi, Aichi-ken, 483(JP)

(74) Representative: **Vossius & Partner**
Siebertstrasse 4 P.O. Box 86 07 67
W-8000 München 86(DE)

(54) Structural gene of membrane-bound alcohol dehydrogenase complex,plasmid containing the same and transformed acetic acid bacteria.

(57) There is provided a structural gene of membrane-bound alcohol dehydrogenase complex having a molecular size of about 7.0 kilo base which is derived from a microorganism belonging to the genus Acetobacter represented by Acetobacter altoacetigenes and shown by the nucleotide sequence of Fig. 3 and Fig. 4. This enzyme increases the efficiency of acetic acid fermentation and may be effectively utilized for quantitative determination of alcohol.

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The present invention relates to a structural gene of membrane-bound alcohol dehydrogenase complex derived from a microorganism belonging to the genus Acetobacter, and a plasmid containing the same as well as its utilization.

A membrane-bound alcohol dehydrogenase produced by a microorganism belonging to the genus Acetobacter is an enzyme which oxidizes an alcohol into the corresponding acid . The enzyme takes a part in oxidative fermentation of acetic acid fermentation for producing acetic acid from ethanol, and is also utilized for quantitative determination of alcohol; the enzyme is useful from an industrial viewpoint.

Heretofore the membrane-bound alcohol dehydrogenase (hereafter simply referred to as ADH) has been obtained by culturing a microorganism belonging to the genus Acetobacter or the genus Gluconobacter, extracting and purifying from the cultured cells and has been utilized (Agricultural and Biological Chemistry, 42, 2045, 1978; ibid., 42, 2331, 1978).

For purification of this enzyme, however, fractionation by complicated column chromatography was required so that it was difficult to prepare the enzyme in large quantities. In addition, the enzyme is unstable and cannot be stored over a long period of time, which has been a problem in its application.

In order to solve these problems, it is considered to harvest mutants having an enhanced enzyme content in the cells by a mutation treatment. However, there is no report yet that any mutant having a sufficient enzyme content was harvested. It is also considered to achieve the object by cloning a gene of the enzyme and increasing the copy number of the enzyme gene or enhancing an expression degree, through genetic engineering technology. For this attempt, ADH gene of Acetobacter aceti K6033 strain has been cloned and its nucleotide sequence has been determined (Journal of Bacteriology, 171, 3115, 1989). This study is expected to be effective for improving the productivity of the enzyme by genetic engineering technology. In actuality, however, even though a plasmid carrying the enzyme gene is introduced into a host of acetic acid bacteria, the enzyme activity is not improved more than the activity inherently possessed by the host and such technique is not practical.

This is believed to be because the cloned gene would be composed only of subunits having a larger molecular weight out of the subunits constructing ADH. Any conventional ADH is purified in the form of a complex with cytochrome c from acetic acid bacteria. Matsushita et al. reported that the activity of ADH was affected depending upon the quantity of cytochrome c and cytochrome c was not present merely as an impurity but took a part in expressing the enzyme activity (Agricultural and Biological Chemistry, 53, 2895, 1989). For this reason, it was necessary to increase the subunits having a large molecular weight and at the same time, increase the amount of the subunits of cytochrome c.

Furthermore, properties of the enzyme in the cloned Acetobacter aceti K6033 strain were not studied and utility of the enzyme of K6033 strain is unclear.

In order to solve the foregoing problems, the present inventors have brought attention to ADH produced by a series of microorganisms belonging to the genus Acetobacter represented by Acetobacter altoacetigenes, which are already known to have enzymatically excellent properties, and have succeeded in cloning the structural gene of two proteins (subunits) constructing ADH and in carrying the structural gene on a plasmid.

Furthermore, the present inventors have found that by using the plasmid carrying the cloned gene, the content of this enzyme in the cells can be increased, ADH can be readily extracted and purified, and the efficiency of acetic acid fermentation can be improved. The present invention has thus been accomplished.

The present invention relates to a structural gene of ADH complex which is derived from a microorganism belonging to the genus Acetobacter, has the restriction map given in Fig. 1 and has a length of about 7.0 kilobase pairs (kb). The invention further relates to a plasmid carrying the gene as well as a microorganism belonging to the genus Acetobacter or the genus Gluconobacter transformed with the plasmid. The present invention further relates to a structural gene of a protein constituting an ADH complex, which is represented by the nucleotide sequence shown in Fig. 3 and has a molecular weight of about 72,000, and a plasmid carrying the gene as well as a microorganism belonging to the genus Acetobacter or the genus Gluconobacter transformed with the plasmid. The present invention also relates to a structural gene of a protein constituting an ADH complex, which is represented by the nucleotide sequence shown in Fig. 4 and has a molecular weight of about 44,000, and a plasmid carrying the gene as well as a microorganism belonging to the genus Acetobacter or the genus Gluconobacter transformed with the plasmid.

Fig. 1 shows the restriction map of the structural gene of a membrane-bound alcohol dehydrogenase complex isolated using Pst I.

Fig. 2 shows the restriction enzyme map of the structural gene of a protein having a molecular weight of about 72,000 which constitutes a membrane-bound alcohol dehydrogenase complex isolated using Sma I.

Fig. 3: the upper lines show the nucleotide sequence of the structural gene of a protein having a molecular weight of about 72,000 which constitutes a membrane-bound alcohol dehydrogenase complex isolated using Sma I, and the lower lines show the amino acid sequence determined from the nucleotide sequence of the structural gene of a membrane-bound alcohol dehydrogenase complex.

Fig. 4: the upper lines show the nucleotide sequence of the structural gene of a protein having a molecular weight of about 44,000 which constitutes a membrane-bound alcohol dehydrogenase complex isolated using Pst I, and the lower lines show the amino acid sequence determined from the nucleotide sequence of the structural gene of a membrane-bound alcohol dehydrogenase complex. Abbreviations in the amino acid sequences have the following meaning:

Met	methionine	Ala	alanine
Arg	arginine	Asn	asparagine
Asp	aspartic acid	Cys	cystein
Gln	glutamine	Glu	glutamic acid
Gly	glycine	His	histidine
Ile	isoleucine	Leu	leucine
Lys	lysine	Phe	phenylalanine
Pro	proline	Ser	serine
Thr	threonine	Trp	tryptophan
Tyr	tyrosine	Val	valine

The membrane-bound alcohol dehydrogenase complex in the present invention refers to a novel alcohol dehydrogenase complex having excellent stability which is described in Japanese Patent Application Laid-Open No. 63-12278 and composed of two proteins having molecular weights of about 72,000 and about 44,000. This enzyme is produced by a series of microorganisms belonging to the genus Acetobacter represented by Acetobacter altoacetigenes MH-24 (FERM BP-491).

The gene fragment containing the structural gene of the enzyme can be cloned from the total DNA which the microorganism belonging to the genus Acetobacter capable of producing this enzyme carries.

The total DNA may be prepared by, for example, the method disclosed in Japanese Patent Application Laid-Open No. 60-9489. The gene fragment containing the structural gene of the ADH complex may be cloned from the total DNA by, for example, the procedures shown in Example 1, that is, determining a part of the amino acid sequence of this enzyme, preparing synthetic DNA corresponding to the determined amino acid sequence and selecting a clone having the desired gene utilizing the synthetic DNA as a probe; etc. The amino acid sequence may be determined as follows: after the alcohol dehydrogenase complex purified by the method disclosed in Japanese Patent Application Laid-Open No. 63-12278 is separated into two subunits by SDS-polyacrylamide gel electrophoresis, the protein corresponding to each subunit is extracted from the gel in a conventional manner such as electric dialysis, etc. The extracted protein is used for determination of amino acid sequence at the amino terminus as it is. Alternatively, after the protein is cleaved with CNBr or with a protease (peptidase) having a high specificity, the cleavage product is fractionated by gel filtration, etc. and the resulting fraction is used for determination of amino acid sequence at the amino terminus in a conventional manner using an amino acid sequencer, etc. Synthesis of DNA corresponding to the thus determined amino acid sequence may be carried out in a conventional manner.

An antibody to the enzyme may be prepared by separating into two subunits the alcohol dehydrogenase complex purified by the method disclosed in Japanese Patent Application Laid-Open No. 63-12278 by SDS-polyacrylamide gel electrophoresis, extracting the protein corresponding to each subunit from the gel in a conventional manner such as electric dialysis, etc. and using the extracted protein as an antigen. Specifically, anti-ADH antibody may be obtained by, for example, the method described in

"Methods in Enzymology", 73, 46 (1981). About 2 weeks after the first immunization, a second immunization is made and in a month to a month and a half, the production of the antibody specific to ADH is observed. This antibody may be further purified either through purification by ammonium sulfate fractionation, etc. or by ion exchange chromatography. In the case that the antibody is used to clone the gene, it may also be possible to use appropriately diluted serum.

On the other hand, the cleavage product of the total DNA with an appropriate restriction enzyme is ligated with the cleavage product of an appropriate vector with a restriction enzyme capable of ligating with the total DNA using T4 DNA ligase. The ligation product is transformed to E. coli host. Examples of the vector used in this case include vectors of E. coli generally used, such as pBR322, pUC18, pUC19, and the like.

Transformation of E. coli may be conducted in a conventional manner. Detection of a strain bearing the desired gene can be made by preparing synthetic DNA based on the amino acid sequence previously determined using the purified enzyme and performing conventional colony hybridization using the synthetic DNA as a probe, whereby a strain reactive with the probe is selected.

Also where antigen-antibody reaction is utilized, a strain carrying the desired gene may be detected by a method similar to, e.g., Gene, 37, 267 (1985). That is, the lysate of the resulting transformants is reacted with the antibody and a strain showing a specific reaction may be selected.

The strain selected by the procedures described above may have a plasmid carrying the gene fragment having the entire length of the desired gene but may sometimes carry merely a part of the gene.

Where the strain has merely a part of the gene, the entire length of the gene may be obtained by using as a probe the gene already obtained and isolating a fraction showing homology to the probe by Southern hybridization, etc.

The nucleotide sequence of the resulting gene may be determined in a conventional manner, for example, by the dideoxy method using M13 phage.

In order to produce the ADH complex or the proteins constructing the ADH complex using the thus isolated gene fragment containing the structural gene of the ADH complex, in general, it is necessary to ligate the gene fragment carrying the enzyme gene with a gene having a promoter activity functioning in a host in the form of capable of expression. As the promoter used to produce the ADH complex proteins in a microorganism belonging to the genus Acetobacter or the genus Gluconobacter, there may be used a promoter inherently possessed by the ADH complex gene and there may also be used an acetic acid bacteria-derived gene having other promoter activity and a promoter of E. coli capable of expressing in acetic acid bacteria. As the E. coli promoter, there may be used promoters of ampicillin-resistant gene of E. coli plasmid pBR322, kanamycin-resistant gene of E. coli plasmid pACYC177, chloramphenicol-resistant gene of E. coli plasmid pACYC184, B-galactosidase gene of E. coli, etc. Where the ADH complex is produced in an excess amount to affect growth or the like of the host, it is necessary to choose an appropriate promoter for controlling an expression amount of the gene. Where the gene is expressed, formation of a protein having a size different from the molecular weight of the gene is sometimes observed. This is because the protein is produced in a host in the form of a fused protein in which other protein is fused. However, if the fused protein is produced in such a form that its enzyme activity can be expressed, there would be no problem.

As the vector for carrying the gene fragment containing the structural gene of the ADH complex in acetic acid bacteria, there may be utilized, for example, pTA5001(A) and pTA5001(B) disclosed in Japanese Patent Application Laid-Open No. 60-9488; wide host range vectors RP4::Mu, RP4, pRK2013, RSF1010 etc. which can be introduced into acetic acid bacteria.

For expression of the activity of ADH, it is necessary that the two proteins constituting the ADH complex be produced efficiently with good balance, as shown in the EXAMPLES. In general, the gene fragment containing the structural gene of the ADH complex is used as it is and the two proteins may be expressed on the same level. Depending upon acetic acid bacteria, however, either protein is not sufficiently possessed in some occasion. In this case, it is required that the gene encoding the two proteins are independently prepared and the genes having a promoter activity used to express the genes are selected to be a suitable expression amount, respectively. For controlling the expression amount, it may also be possible to use different vectors in the two genes and utilize a difference in the copy number of the vectors in acetic acid bacteria.

As stated above, the plasmid containing the structural gene of the ADH complex can be isolated. After transformation, the gene is expressed, whereby the protein constituting the ADH complex can be produced in a marked quantity.

As the host for producing the ADH complex, microorganisms such as E. coli, Bacillus subtilis, etc. on which genetic engineering technique has been established may be used. However, it is more advantageous

to use acetic acid bacteria which inherently possess the ability of producing the ADH complex, namely, the microorganisms belonging to the genus Acetobacter or the genus Gluconobacter.

ADH has pyrroloquinoline quinone (PQQ) as its prosthetic group. In order to produce an activated enzyme, PQQ may be supplemented to a medium, etc. to produce the ADH complex protein. However, as is described in Agricultural & Biological Chemistry, 48, 561 (1984), the ability of E. coli or S. subtilis for synthesizing PQQ is poor and it has been revealed that the synthesizing ability of acetic acid bacteria is high. It is thus advantageous for the host to have the ability for synthesizing PQQ.

Further in acetic acid fermentation, ADH participates in the reaction of oxidizing ethanol to acetaldehyde. For this reason, by enhancing the content of the ADH complex in acetic acid bacteria, it can be expected to make the acetic acid fermentation efficient. In this case, where ADH alone is expressed excessively, the concentration of acetaldehyde, which is the oxidation product of ethanol, increases so that acetic acid bacteria are damaged by strongly cytotoxic acetaldehyde. Therefore, it is necessary either to control the amount of the ADH complex gene expressed to the amount corresponding to the oxidising activity of acetaldehyde or to increase the amount of aldehyde dehydrogenase at the same time, using the structural gene of the membrane-bound aldehyde dehydrogenase recited in Japanese Patent Application Laid-Open No. 63-52709 so as not to cause excessive accumulation of acetaldehyde.

[EXAMPLES]

The present invention is illustrated by the following examples.

EXAMPLE 1

[Determination of amino terminal amino acid sequence and preparation of synthetic probe]

Acetobacter altoacetigenes MH-24 (FERM BP-491) strain was shaking cultured at 30°C in medium composed with 3% of glucose, 4% (V/V) of ethanol, 6% (V/V) of acetic acid, 0.5% of yeast extract (manufactured by Daigo Nutrient Chemistry Co., Ltd.) and 0.2% of polypeptone (manufactued by Daigo Nutrient Chemistry Co., Ltd.).

After the incubation, the cells were harvested by centrifugation and 10 mg of the ADH complex was then obtained in a conventional manner (the method disclosed in Japanese Patent Application Laid-Open No. 63-12278). This complex was subjected to SDS-polyacrylamide gel electrophoresis to separate the protein having a molecular weight of about 72,000 and the protein having a molecular weight of about 44,000. Then, the protein of 72,000 was eluted from the gel in a conventional manner and provided as a sample for the following experiment.

After 1 mg of the sample obtained was cleaved with lysyl endopeptidase (manufactured by Wako Pure Chemicals, Inc.), the cleavage product was fractionated by HPLC LC-4A manufactured by Shimadzu Seisakusho Co., Ltd. As a column Senshu Pak. VP-304-1251 (4.6 ø x 250 mm) was used and the elution was performed at a flow rate of 1 ml/min and at room temperature by linear gradient of acetonitrile-water (containing 0.1% trifluoroacetic acid) of 0 to 55%. By monitoring at absorbance of 220 nm, 11 peaks were noted. From the earlier order of elution, the second, ninth and eleventh peaks were fractionated. About 0.5 mg of the fractionated product was applied to amino acid sequencer Model 470A manufactured by Applied Biosystems Inc. to determine the amino terminal amino acid sequence. The results reveal that the sequence of the peptide eluted in the ninth order was:
Thr-Gly-Leu-Val-Tyr-Ile-Pro-Ala-Gln-Gln-Val-Pro-Phe-Leu-Tyr-Thr-Asn-Gln-Val-Gly-Gly-Phe-Tyr-Pro-His-Pro-Asp; and that the sequence of the peptide eluted in the eleventh order was: Leu-Ala-Trp-Tyr-Leu-Asp-Leu-Asp-Thr-Asn-Arg-Gly-Gln-Glu-Gly-Thr-Pro-Leu. Furthermore, the sequence of the peptide eluted in the second order was: Asn-Tyr-Val-Tyr-Val-Asn-Trp-Ala-Ser-Gly-Leu-Asp-Pro.

The protein having a molecular weight of 72,000 which was not treated with lysyl endopeptidase was analyzed with an amino acid sequencer. The amino terminal amino acid sequence was Asp-Asp-Gly-Gln-Gly. DNA corresponding to the amino acid sequence was synthesized with DNA synthesizer 381A manufactured by Applied Biosystems Inc., based on the two sequences Tyr-Ile-Pro-Ala-Gln-Gln-Val (Sequence 1) and Val-Ile-Ile-Gly-Asn-Gly (Sequence 2) in the amino acid sequence of the peptide eluted in the ninth order and a part of the amino acid sequence, Try-Val-Tyr-Val-Asn-Trp-Ala (sequence 3), in the peptide eluted in the second order, taking utilization of codon into account.

For Sequence 1, Probe 1 a 64-fold degenerate 20-mer was synthesized:

```
   T  T
TA AT CCNGCNCAGCAGCAGG
   C  C
```

For Sequence 2, Probe 2, a 128-fold degenerate 17-mer was synthesized:

```
     T  T    T
GTNAT AT GGNAA GG
     C  C    C
```

For Sequence 3, Probe 3, a 128-fold degenerate 20-mer was synthesized:

```
     A    A  A A
GCCCA TTNAC TAN C TA
     G    G  G G
```

This sequence was derived from the complementary strand.

Cloning of the structural gene of protein having a molecular weight of about 72,000 which constructs the ADH complex

From the cells of Acetobacter altoacetigenes MH-24 strain which had been obtained by culturing as described above, the total DNA was prepared in a conventional manner (the method disclosed in Japanese Patent Application Laid-Open No. 60-9489). After the total DNA was cleaved with a restriction enzyme, Pst I or Sma I (manufactured by Takara Shuzo Co., Ltd.), the product was ligated with E. coli vector pUC18 (manufactured by Takara Shuzo Co., Ltd.) which was cleaved with Pst I or Sma I, and thereafter dephosphorylated with bacterial alkaline phosphatase (manufactured by Takara Shuzo Co., Ltd.), using T4 DNA ligase (manufactured by Takara Shuzo Co., Ltd.). After the ligation mixture was transformed to the host E. coli JM 109 by the method of Hanahan ["DNA Cloning", 1, 109, IRL Press (1985)], transformants were selected in LB agar medium ("A Manual for Genetic Engineering", page 201, Cold Spring Harbor Laboratory, 1980) containing ampicillin in a concentration of 30 μg/ml.

With respect to about 5,000 recombinants obtained, colonies which hybridized with Probe 2 and Probe 3 described above were detected according to the colony hybridization technique ("A Manual for Genetic Engineering", page 312, Cold Spring Harbor Laboratory, 1980) using the two probes. In Pst I, three (3) clones hybridized with Probes 2 and 3,and in Sma I, two (2) clones hybridized with the probes. Furthermore, these 5 clones all hybridized also with Probe 1.

Analysis with restriction enzyme reveals that all of the 3 clones obtained using Pst I had the same fragment of about 7.0 kilo base at the Pst I site of pUC18. Further,in the case of Sma I, the clones had the same fragment of about 4.5 kilo base. The fragment of about 7.0 kilo base obtained with Pst I had a portion of about 4.1 kilo base in common to the fragment of about 4.5 kilo base. The plasmid (chimeric plasmid composed of pUC18 and the insert fragment of about 7.0 kilo base, named pADHP1) possessed by 1 clone obtained using Pst I was transformed in E. coli JM 109 and has been deposited in the Fermentation Research Institute of the Agency of Industrial Science and Technology of Japan under the name of E. coli ADHP-1 as [FERM BP-3254 (FERM P-11278)]. The restriction enzyme map of the insert fragment of about 7.0 kilo base was prepared in a conventional manner, which is as shown in Fig. 1. Furthermore, the plasmid (chimeric plasmid composed of pUC18 and the insert fragment of about 4.5 kilo base, named pADHS1) possessed by 1 clone obtained using Sma I was transformed in E. coli JM 109 and has been deposited in the Fermentation Research Institute of the Agency of Industrial Science and Technology of Japan under the name of E. coli ADHS-1 as [FERM BP-3253 (FERM P-11201)]. The restriction enzyme map of the insert

fragment of about 4.5 kilo base was prepared in a conventional manner, which is as shown in Fig. 2.

With respect to the insert fragment of pADHS1, its nucleotide sequence was determined by the dideoxy method [Methods in Enzymology, 10, 20, Academic Press, New York, 1983] using M13 phage.

Based on the thus determined nucleotide sequence, an open-reading frame was identified. The open-reading frame encoding 738 amino acid residues (molecular weight 80839), composed of 2214 bases and translated from the ATG initiation codon as shown in Fig. 3 was identified in the portion common to the Sma I fragment having a size of about 4.5 kilo base and the Pst I fragment having a size of about 7.0 kilo base (the amino acid sequence determined from the nucleotide sequence of Fig. 3 is shown in Fig. 3, lines below the nucleotide sequence). The polypeptide encoded by the nucleotide sequence of Fig. 3 coincides with the protein having a molecular weight of about 72,000 which constitutes the membrane-bound alcohol dehydrogenase complex of the present invention. This is confirmed by the fact that when the amino acid sequence of the purified protein having a molecular weight of about 72,000, which constructs the membrane-bound alcohol dehydrogenase complex of the present invention, was determined by the method described above, the sequence fully coincident with the amino terminal amino acid sequences of the 3 peptides of the lysyl endopeptidase cleavage products was found. That is, the sequence of 27 amino acids of the peptide eluted in the ninth order coincided with the sequence of 27 amino acids following 442 amino acid from the amino terminus deduced from the nucleotide sequence. Furthermore, the sequence of 18 amino acids of the peptide eluted in the eleventh order coincided with the sequence of 18 amino acids following 84 amino acid deduced from the nucleotide sequence. The amino terminal amino acid sequence of the peptide eluted in the second order coincided with the sequence of 13 amino acids following 389 amino acid deduced from the nucleotide sequence.

Furthermore, the amino terminal sequence (Asp-Asp-Gly-Gln-Gly) of the purified protein completely coincided with the amino acid sequence following the 36th counted from the amino terminus which is deduced from the nucleotide sequence. It is thus assumed that the amino acid sequence up to the 35th from the amino terminus deduced from the nucleotide sequence would be the region which participates in secretion of the protein having a molecular weight of about 72,000. Acetobacter aceti K6033 strain had homology of about 77% to ADH gene in the amino acid sequence.

Preparation of anti-ADH antibody

Acetobacter altoacetigenes MH-24 (FERM BP-491) strain was shakingly cultured at 30° C in medium composed with 3% of glucose, 4% (V/V) of ethanol, 6% (V/V) of acetic acid, 0.5% of yeast extract (manufactured by Daigo Nutrient Chemistry Co., Ltd.) and 0.2% of polypeptone (manufactured by Daigo Nutrient Chemistry Co., Ltd.). After the incubation, the cells were harvested by centrifugation and 4 mg of the ADH complex was then obtained in a conventional manner (the method disclosed in Japanese Patent Application Laid-Open No. 63-12278). This complex was subjected to SDS-polyacrylamide gel electrophoresis to separate the protein having a molecular weight of about 72,000 and the protein having a molecular weight of about 44,000. The respective proteins were eluted from the gel in a conventional manner and provided as samples for the following experiment.

Each 0.1 mg of the samples obtained was subcutaneously injected to rabbit together with complete Freund's adjuvant, and 0.1 mg of each sample was further injected after about 2 weeks. One month after the first injection, rabbit blood was withdrawn from the ear and centrifuged. The reactivity of the thus obtained serum with the two proteins was examined, whereby precipitation was noted. Further after SDS-polyacrylamide gel electrophoresis, its specificity was examined by Western blotting, using the cell-free extract of Acetobacter altoacetigenes MH-24 and E. coli JM 109. Reactivity with proteins other than the objective protein was not appreciable but the antibody having high specificity was produced.

Cloning of gene containing the full length of the structural gene of ADH complex

The Pst I fragment having a size of about 7.0 kilo base containing the structural gene of the protein having a molecular weight of 72,000, which constructed the ADH complex, obtained by the procedures described above and the Sma I fragment having a size of about 4.5 kilo base were ligated at the Pst I site or Sma I site of E. coli vector pUC18, respectively, in a conventional manner. The ligated chimeric plasmid was transformed to E. coli JM 109 in a conventional manner to give transformants carrying the chimeric plasmids. From the transformants, the plasmids were prepared in a conventional manner followed by analysis with restriction enzymes.

By the analysis with restriction enzymes, selection was made for chimeric plasmids in which the Pst I fragment or the Sma I fragment was inserted in such a way that the transcription direction of the lac

promoter of E. coli vector pUC18 was the same as the transcription direction of the structural gene of the protein having a molecular weight of about 72,000, which constructed the ADH complex. The transformants carrying these plasmids were cultured at 37°C for 8 hours in LB medium containing 30 μg/ml of ampicillin and 1 mM of isopropyl-β-thiogalactopyranoside (IPTG). The cells were sonicated, and the resulting homogenate was subjected to SDS-polyacrylamide gel electrophoresis. A molecular weight of the protein specifically reacting with the antibody was determined using an antibody capable of specifically reacting with the two proteins which constructed the ADH complex described above, according to the Western blotting method (Annal. Biochem., 12, 195 (1981)). When detection was made using the antibody to the protein having a molecular weight of about 72,000, the reaction with the protein having a molecular weight of about 72,000 was noted both in the case of carrying the Pst I fragment and in the case of carrying the Sma I fragment. In the transformant carrying vector pUC18 alone which was used for control, no protein capable of reacting with the antibody was detected. By the foregoing, it was confirmed that the structural gene of the protein having a molecular weight of about 72,000 was present on the Pst I fragment and on the Sma I fragment.

On the other hand, detection was made using the antibody to the protein having a molecular weight of about 44,000. In the transformant carrying only vector pUC18 that was used for control, no protein capable of reacting with the antibody was noted. However, in the transformant carrying the plasmid into which the Sma I fragment had been inserted, the reaction with the protein having a molecular weight of about 24,000 was noted. Further in the transformant carrying the plasmid into which the Pst I fragment had been inserted, the reaction with the protein having a molecular weight of about 44,000 was noted. To the contrary, in the cells cultured in liquid medium containing no IPTG, the protein having a molecular weight of about 44,000 and capable of reacting with the antibody was not detected.

These results indicate that the structural gene encoding the protein having a molecular weight of about 44,000, which is cytochrome c, is present on the Pst I fragment and the direction of its transcription is the same as that of the protein having a molecular weight of about 72,000. From the fact that the molecular weight is about 44,000, it is also assumed that the region of the structural gene necessary for encoding this protein would be about 1.2 kilo base. Taking the size of the protein capable of reacting with the antibody into account, it is assumed that the structural gene of cytochrome c having a molecular weight of about 44,000 would be present immediately downstream the structural gene of the protein having a molecular weight of about 72,000 and transcribed and expressed in one unit.

Based on the foregoing results, it was confirmed that the structural genes of the protein having a molecular weight of about 72,000 and the protein having a molecular weight of about 44,000 are present on the gene fragment cleaved with Pst I in the restriction enzyme map shown in Fig. 1.

EXAMPLE 2

Transformation of the gene fragment containing the structural gene of ADH complex into acetic acid bacteria host

Chimeric plasmid pADHS1 of the Sma I fragment (about 4.5 kilo base) containing the structural gene of the protein having a molecular weight of about 72,000, which constructed the ADH complex isolated in EXAMPLE 1 was extracted from E. coli ADHS-1 in a conventional manner to give purified DNA. After 1 μg of this DNA was cleaved with Sac I, the cleavage end was rendered blunt with T4 DNA polymerase. On the other hand, plasmid named pTA5001 was prepared from Acetobacter aceti No. 1023 [FERM BP-2287 (FERM P-7122)] according to the method described in Agricultural and Biological Chemistry, 49, 1011 (1985) (pTA5001 is described in Agricultural and Biological Chemistry, 49, 1011 (1985). pTA5001 is a mixture of two plasmids of pTA500IA having a length of 23.5 kilo base and pTA500IB having a length of 23 kilo base.). After 5 μg of this plasmid DNA werecleaved with Xho I, the cleavage end was rendered blunt with T4 DNA polymerase.

The cleaved DNAs of pADHS1 and pTA5001 prepared as described above were ligated with each other using T4 DNA ligase to give the ligation product. Thereafter, the product was transformed in ADH activity-deleted mutant 10-80 according to the method described in Agricultural and Biological Chemistry, 49, 2091 (1985). The transformants were selected in YPG agar medium (3% of glucose, 0.5% of yeast extract, 0.2% of polypeptide, 2% of agar, pH 6.5) containing 50 μg/ml of ampicillin. Plasmids of 10 ampicillin-resistant strains grown in the selection medium were analyzed by a modified method of Agricultural and Biological Chemistry, 49, 2083 (1985). As the result, the size of the plasmids introduced were all about 31 kilo base. Analysis with restriction enzymes reveals that they were all chimeric plasmid of three components: pUC18, The Sma I fragment of 4.5 kilo base containing the structural gene of the protein having a molecular weight

of about 72,000 which constructed the ADH complex, and pTA5001. This chimeric plasmid was named pMADHSI.

After pADHPI was cleaved with Sac I as in pADHSI, chimeric plasmid of plasmid pADHP1 isolated in EXAXPLE 1 and pTA500I was prepared in a manner similar to the case of pADHSI. The chimeric plasmid was transformed into mutant 10-80 of Acetobacter aceti No. 1023 to give the transformant carrying the chimeric plasmid (named pMADHPI).

Properties of acetic acid bacteria transformant

With respect to the two transformants of mutant 10-80 of Acetobacter aceti No. 1023 obtained above, enzyme activity of ADH was assayed. Firstly, ampicillin was added to YPG liquid medium (medium having a composition obtained by removing agar from YPG agar medium described above) in a concentration of 30 μg/ml followed by shaking-culture at 30°C for 36 hours. After culturing, the cells were harvested, suspended in McIlvaine buffer (pH 6.0) and homogenized with a French press. ADH activity in the supernatant obtained from the homogenate was measured by a method of Agricultural and Biological Chemistry, 49, 2045 (1978). At the same time, aldehyde dehydrogenase (ALDH) activity was also determined by a method of Agricultural and Biological Chemistry, 44, 503 (1980). These results are shown in Table 1.

Table 1

Strain	Chimera Plasmid Carried	Enzyme Activity (U/mg protein) ADH	ALDH
No. 1023	none	0.28	0.94
10-80	none	0.01	0.85
10-80	pMADHS1	0.01	0.90
10-80	pMADHP1	0.40	1.00

Mutant 10-80 obtained from Acetobacter aceti No. 1023 is a strain which is specifically deleted of ADH activity. The transformant of this strain carrying plasmid pMADHSI containing the structural gene alone encoding the protein having a molecular weight of about 72,000 did not show ADH activity yet. On the other hand, in the transformant carrying plasmid pMADHPI concurrently containing the gene encoding the protein having a molecular weight of about 44,000, restoration of ADH activity was noted. From the results, it is shown that for expression of ADH activity, two proteins having a molecular weight of 72,00 and a molecular weight of 44,000 which construct the ADH complex are required.

It is also noted that the specific activity of the parent having no chimeric plasmid was 0.28 (unit/mg protein), whereas the the specific activity of transformant was 0.40, showing an increase of the activity by about 1.4 times.

As described above, the cell content of ADH having the activity can be increased by transforming acetic acid bacteria with the gene containing the structural gene of ADH complex.

EXAMPLE 3

Determination of nucleotide sequence of the structural gene of the protein having a molecular weight of about 44,000 which constructs the ADH complex

The results of EXAMPLE 1 reveal that the structural gene encoding the protein having a molecular weight of about 44,000 is present right downstream of the structural gene encoding the protein having a molecular weight of about 72,000. Therefore, the nucleotide sequence of an about 2.8 kilo base fragment containing the region downstream of the structural gene encoding a protein having a molecular weight of about 72,000 in the insert fragment of pADHPI, restriction enzyme map of which is shown in Fig. 1 (from the left Cla I site to the right BamH I site)

was determined by the dideoxy method (Methods in Enzymology, 10, 20, Academic Press, New York,

1983), using M13.

Based on the determined nucleotide sequence, the open-reading frame which could encode the protein having a molecular weight of about 44,000 downstream of the nucleotide sequence shown in Fig. 3 was analyzed and an open-reading frame which could encode a protein of 468 amino acid residues (molecular weight of 49757) composed of 1404 bases starting with translation initiation codon ATG as shown in Fig. 4, was found (the amino acid sequence determined from the nucleotide sequence in Fig. 4 is shown in Fig. 4 below the nucleotide sequence). In order to confirm that the polypeptide having the amino acid sequence shown in Fig. 4 coincides with the protein having a molecular weight of about 44,000 which constitutes the membrane-bound alcohol dehydrogenase complex of the present invention, the protein having a molecular weight of about 44,000 was isolated from the membrane-bound alcohol dehydrogenase complex. The protein was treated with Lysyl endopeptidase, the resulting cleavage product was fractionated and the amino terminal amino acid sequence of the resulting peptide was determined, in a manner similar to EXAMPLE 1. It is confirmed that the same amino acid sequence as that determined is present in the sequence shown in Fig. 4. That is, lysyl endopeptidase was acted on the protein having a molecular weight of about 44,000 isolated in a manner similar to EXAMPLE 1. The resulting cleavage product was fractionated by HPLC in a manner similar to EXAMPLE 1. Among the eluted peptides, the first and fourth peptides were fractionated in the earlier order of elution. Using about 0.1 mg of the fractionated product, the amino acid sequence at the amino terminus was determined in a manner similar to EXAMPLE 1. As the result, the amino terminal amino acid sequence of the peptide firstly eluted was determined to be Asp-Phe-Tyr-Pro-Ala-Pro and the amino terminal amino acid sequence of the peptide fourthly eluted was determined to be Ser-Leu-Ser-Ala-Glu-Glu.

These sequences coincided with the sequence after 169 and with the sequence after 390, from the amino terminus, in the amino acid sequence shown in the lower lines in Fig. 4. It was thus confirmed that the gene having the nucleotide sequence shown in Fig. 4 was the structural gene of the protein having a molecular weight of about 44,000 which constituted the ADH complex.

According to the present invention, the structural gene of the ADH complex produced by a series of microorganisms belonging to the genus Acetobacter represented by Acetobacter altoacetigenes can be cloned and the structural gene can be successfully incorporated into a plasmid. Further by using acetic acid bacteria transformed by the plasmid, efficiency of acetic acid fermentation can be increased. Moreover, the ADH complex can be readily extracted and purified from the acetic aced bacteria and this enzyme can be utilized for quantitative determination of alcohol.

While the invention has been described in detail and with reference to specific embodiments thereof, it is apparent to one skilled in the art that various changes and modifications can be made therein without departing from the spirit and the scope of the present invention.

1. SEQ ID NO: 1:

SEQUENCE TYPE:	nucleic acid
SEQUENCE LENGTH:	2214 bp
STRANDEDNESS:	double
MOLECULE TYPE:	genomic DNA
ORIGINAL SOURCE	
ORGANISM:	Acetobacter altoacetigenes MH-24

```
ATGATTTCTG CCGTTTTCGG AAAAAGACGT TCTCTGAGCA GAACGCTTAC AGCCGGAACG    60
ATATGTGCGG CTCTCATCTC CGGGTATGCC ACCATGGCAT CCGCAGATGA CGGGCAGGGC   120
GCCACGGGGG AAGCGATCAT CCATGCCGAT GATCACCCCG GTAACTGGAT GACCTATGGC   180
CGCACCTATT CTGACCAGCG CTACAGCCCG CTGGATCAGA TCAACCGTTC CAATGTCGGT   240
AACCTGAAGC TGGCCTGGTA TCTGGACCTT GATACCAACC GTGGCCAGGA AGGCACGCCC   300
CTGGTTATTG ATGGCGTCAT GTACGCCACC ACCAACTGGA GCATGATGAA AGCCGTCGAC   360
GCCGCAACCG GCAAGCTGCT GTGGTCCTAT GACCCGCGCG TGCCCGGCAA CATTGCCGAC   420
AAGGGCTGCT GTGACACGGT CAACCGTGGC GCGGCATACT GGAATGGCAA GGTCTATTTC   480
GGCACGTTCG ACGGTCGCCT GATCGCGCTG GACGCCAAGA CCGGCAAGCT GGTCTGGAGC   540
GTCAACACCA TTCCGCCCGA AGCGGAACTG GGCAAGCAGC GTTCCTATAC GGTTGACGGC   600
GCGCCCCGTA TCGCCAAGGG CCGCGTGATC ATCGGTAACG GTGGTTCCGA ATTCGGTGCC   660
CGTGGCTTCG TCAGCGCGTT CGATGCGGAA ACCGGCAAGG TCGACTGGCG CTTCTTCACG   720
GTTCCGAACC CCAAGAACGA ACCGGACGCT GCATCCGACA GCGTGCTGAT GAACAAGGCC   780
TACCAGACCT GGAGCCCGAC CGGCGCCTGG ACCCGCCAGG GTGGCGGCGG CACGGTATGG   840
GATTCCATCG TGTATGACCC CGTGGCCGAC CTGGTCTACC TGGGCGTTGG CAACGGTTCG   900
CCGTGGAACT ACAAGTACCG TTCCGAAGGC AAGGGCGACA ACCTGTTCCT GGGCAGCATC   960
GTCGCACTGA AGCCGGAAAC CGGCGAATAC GTCTGGCATT TCCAGGAAAC GCCGATGGAC  1020
CAGTGGGACT TCACCTCGGA CCAGCAGATC ATGACGCTTG ACCTGCCGAT CAATGGTGAA  1080
ACCCGCCACG TCATCGTCCA TGCGCGCAAG AACGGCTTCT TCTACATCAT CGATGCGAAG  1140
ACCGGTGAGT TCATCTCGGG CAAGAACTAC GTCTATGTGA ACTGGGCCAG CGGCCTTGAT  1200
CCCAAGACCG GCCGTCCGAT CTACAACCCC GATGCGCTCT ACACCCTTAC GGGCAAGGAA  1260
TGGTACGGCA TTCCGGGTGA CCTTGGCGGC CATAACTTCG CGGCCATGGC GTTCAGCCCC  1320
AAGACCGGGC TGGTCTATAT TCCGGCGCAG CAGGTTCCGT TCCTGTACAC CAATCAGGTC  1380
GGTGGCTTCA CGCCGCACCC CGACAGCTGG AACCTGGGTC TGGACATGAA CAAGGTCGGT  1440
ATTCCCGACT CGCCTGAAGC CAAGCAGGCC TTCGTGAAGG ACCTGAAGGG CTGGATCGTG  1500
GCCTGGGATC CGCAGAAGCA GGCTGAAGCA TGGCGCGTGG ACCACAAGGG GCCGTGGAAC  1560
GGCGGTATCC TGGCAACTGG CGGCGACCTG CTGTTCCAGG GCTTGGCGAA CGGCGAATTC  1620
CATGCCTATG ACGCGACGAA CGGTTCCGAC CTGTTCCACT TCGCGGCGGA CAGCGGCATC  1680
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ATCGCACCGC CTGTGACCTA CCTTGCCAAT GGCAAGCAGT ATGTTGCGGT TGAAGTGGGC   1740
TGGGGCGGCA TCTATCCGTT CTTCCTTGGT GGCCTGGCCC GTACCAGCGG CTGGACCGTC   1800
AACCACTCGC GCATCATTGC CTTCTCGCTC GATGGCAAGT CCGGCCCGCT GCCCAAGCAG   1860
AATGACCAGG GCTTCCTGCC CGTCAAGCCG CCGGCACAGT TCGACAGCAA GCGTACCGAT   1920
AACGGTTACT TCCAGTTCCA GACCTATTGC GCCGCCTGTC ATGGCGATAA CGCAGAAGGT   1980
GCCGGTGTGC TGCCTGACCT GCGCTGGTCC GGGTCCATCC GTCATGAGGA CGCGTTCTAC   2040
AATGTTGTCG GCCGCGGCGC GCTTACCGCC TACGGTATGG ATCGCTTGCA CGGTAACATG   2100
AACCCGACCG AGATTGAGGA CATCCGCCAG TTCCTGATCA AGCGTGCGAA CGAGACCTAT   2160
CAGAGGGAAG TTGATGCCCG GAAGAACGCT GACGGTATCC CCGAGCAGCT GCCG          2214
```

2. SEQ ID NO: 2:

SEQUENCE TYPE:	nucleic acid
SEQUENCE LENGTH:	1404 bp
STRANDEDNESS:	double
MOLECULE TYPE:	genomic DNA
ORIGINAL SOURCE ORGANISM:	Acetobacter altoacetigenes MH-24

```
ATGATCAACA GACTTAAGGT GACATTCAGC GCGGCAGCGT TTAGTCTGCT GGCAGGGACG    60
GCATTGGCAC AGACGCCAGA TGCTGACTCC GCGCTGGTCC AGAAGGGGGC ATATGTCGCG   120
CGACTGGGTG ACTGCGTAGC ATGTCATACC GCTCTCCATG GACAGTCGTA CGCAGGCGGG   180
CTTGAAATCA AGAGCCCGAT CGGTACGATC TACTCCACGA ACATCACACC GGACCCGACC   240
TACGGTATCG GTCGCTACAC CTTCGCCGAA TTCGACGAAG CCGTGCGCCA TGGTATCCGC   300
AAGGACGGTT CCACGCTGTA TCCGGCCATG CCGTATCCCT CCTTCTCGCG CATGACGAAG   360
GAAGACATGC AGGCGCTGTA TGCGTACTTC ATGCATGGGG TGAAGCCGGT CGCGCAGCCG   420
GACAAGCAGC CGGACATCTC CTGGCCCTTG TCCATGCGCT GGCCGCTGGG CATCTGGCGC   480
ATGATGTTCT CGCCTTCGCC GAAGGACTTC ACGCCGGCGC CAGGCACGGA TCCTGAAATC   540
GCACGTGGCG ATTATCTGGT TACCGGCCCC GGGCATTGCG GTGCGTGTCA TACCCCGCGT   600
GGCTTCGCCA TGCAGGAAAA GGCGCTGGAC GCTGCCGGTG GTCCTGACTT CCTGTCCGGT   660
GGCGCACCGA TCGACAACTG GGTCGCGCCG AGCCTGCGCA ACGATCCTGT CGTTGGTCTG   720
GGCCGCTGGT CCGAGGATGA CATCTACACC TTCCTGAAGT CCGGCCGTAT CGACCACTCC   780
GCCGTGTTCG GTGGCATGGG CGATGTGGTG GCATGGAGCA CCCAGTACTT CACCGATGAC   840
GACCTGCACG CCATCGCGAA GTACCTGAAG AGCCTGCCGC CGGTGCCGCC GTCACAGGGC   900
AACTACACCT ACGATCCGTC CACCGCGAAC ATGCTGGCTT CGGGTAATAC CGCCAGCGTT   960
CCGGGTGCTG ATACGTATGT GAAGGAATGC GCCATCTGTC ACCGTAACGA CGGTGGTGGC  1020
GTGGCCCGCA TGTTCCCGCC GCTGGCTGGC AACCCGGTTG TCGTGACCGA GAACCCGACC  1080
TCGCTGGTGA ACGTGATTGC GCATGGTGGC GTGCTGCCGC CGAGCAACTG GGCACCGTCC  1140
GCAGTGGCAA TGCCGGGTTA CAGCAAGTCG CTGTCCGCCC AGCAGATTGC TGATGTGGTC  1200
AACTTCATCC GCACCAGCTG GGGCAACAAG GCGCCCGGCA CCGTTACGGC TGCGGATGTT  1260
ACCAAGCTGC GCGACACGGG CGCCCCGGTT TCCAGCTCTG GCTGGAACAG CGTGAGCAGC  1320
GGCTGGTCGG TCTTCCTGCC GCAGCCTTAC GGCTCGGGCT GGACGTTTGC CCCGCAGACG  1380
CACACCGGTC AGGACGCCGC ACAG                                          1404
```

3. SEQ ID NO: 3:

SEQUENCE TYPE:	amino acid
SEQUENCE LENGTH:	738
MOLECULE TYPE:	protein
ORIGINAL SOURCE	
ORGANISM:	Acetobacter altoacetigenes MH-24
FEATURES:	The mature peptide consists of the amino acids at positions 36 to 738.

```
Met Ile Ser Ala Val Phe Gly Lys Arg Arg Ser Leu Ser Arg Thr Leu
 1               5                  10                  15

Thr Ala Gly Thr Ile Cys Ala Ala Leu Ile Ser Gly Tyr Ala Thr Met
            20                  25                  30

Ala Ser Ala Asp Asp Gly Gln Gly Ala Thr Gly Glu Ala Ile Ile His
        35                  40                  45

Ala Asp Asp His Pro Gly Asn Trp Met Thr Tyr Gly Arg Thr Tyr Ser
    50                  55                  60

Asp Gln Arg Tyr Ser Pro Leu Asp Gln Ile Asn Arg Ser Asn Val Gly
65                  70                  75                  80

Asn Leu Lys Leu Ala Trp Tyr Leu Asp Leu Asp Thr Asn Arg Gly Gln
                85                  90                  95

Glu Gly Thr Pro Leu Val Ile Asp Gly Val Met Tyr Ala Thr Thr Asn
            100                 105                 110

Trp Ser Met Met Lys Ala Val Asp Ala Ala Thr Gly Lys Leu Leu Trp
        115                 120                 125

Ser Tyr Asp Pro Arg Val Pro Gly Asn Ile Ala Asp Lys Gly Cys Cys
    130                 135                 140

Asp Thr Val Asn Arg Gly Ala Ala Tyr Trp Asn Gly Lys Val Tyr Phe
145                 150                 155                 160

Gly Thr Phe Asp Gly Arg Leu Ile Ala Leu Asp Ala Lys Thr Gly Lys
                165                 170                 175

Leu Val Trp Ser Val Asn Thr Ile Pro Pro Glu Ala Glu Leu Gly Lys
            180                 185                 190

Gln Arg Ser Tyr Thr Val Asp Gly Ala Pro Arg Ile Ala Lys Gly Arg
        195                 200                 205

Val Ile Ile Gly Asn Gly Gly Ser Glu Phe Gly Ala Arg Gly Phe Val
    210                 215                 220

Ser Ala Phe Asp Ala Glu Thr Gly Lys Val Asp Trp Arg Phe Phe Thr
225                 230                 235                 240

Val Pro Asn Pro Lys Asn Glu Pro Asp Ala Ala Ser Asp Ser Val Leu
                245                 250                 255

Met Asn Lys Ala Tyr Gln Thr Trp Ser Pro Thr Gly Ala Trp Thr Arg
            260                 265                 270

Gln Gly Gly Gly Gly Thr Val Trp Asp Ser Ile Val Tyr Asp Pro Val
        275                 280                 285

Ala Asp Leu Val Tyr Leu Gly Val Gly Asn Gly Ser Pro Trp Asn Tyr
    290                 295                 300
```

Lys Tyr Arg Ser Glu Gly Lys Gly Asp Asn Leu Phe Leu Gly Ser Ile
305 310 315 320

Val Ala Leu Lys Pro Glu Thr Gly Glu Tyr Val Trp His Phe Gln Glu
325 330 335

Thr Pro Met Asp Gln Trp Asp Phe Thr Ser Asp Gln Gln Ile Met Thr
340 345 350

Leu Asp Leu Pro Ile Asn Gly Glu Thr Arg His Val Ile Val His Ala
355 360 365

Arg Lys Asn Gly Phe Phe Tyr Ile Ile Asp Ala Lys Thr Gly Glu Phe
370 375 380

Ile Ser Gly Lys Asn Tyr Val Tyr Val Asn Trp Ala Ser Gly Leu Asp
385 390 395 400

Pro Lys Thr Gly Arg Pro Ile Tyr Asn Pro Asp Ala Leu Tyr Thr Leu
405 410 415

Thr Gly Lys Glu Trp Tyr Gly Ile Pro Gly Asp Leu Gly Gly His Asn
420 425 430

Phe Ala Ala Met Ala Phe Ser Pro Lys Thr Gly Leu Val Tyr Ile Pro
435 440 445

Ala Gln Gln Val Pro Phe Leu Tyr Thr Asn Gln Val Gly Gly Phe Thr
450 455 460

Pro His Pro Asp Ser Trp Asn Leu Gly Leu Asp Met Asn Lys Val Gly
465 470 475 480

Ile Pro Asp Ser Pro Glu Ala Lys Gln Ala Phe Val Lys Asp Leu Lys
485 490 495

Gly Trp Ile Val Ala Trp Asp Pro Gln Lys Gln Ala Glu Ala Trp Arg
500 505 510

Val Asp His Lys Gly Pro Trp Asn Gly Gly Ile Leu Ala Thr Gly Gly
515 520 525

Asp Leu Leu Phe Gln Gly Leu Ala Asn Gly Glu Phe His Ala Tyr Asp
530 535 540

Ala Thr Asn Gly Ser Asp Leu Phe His Phe Ala Ala Asp Ser Gly Ile
545 550 555 560

Ile Ala Pro Pro Val Thr Tyr Leu Ala Asn Gly Lys Gln Tyr Val Ala
565 570 575

Val Glu Val Gly Trp Gly Gly Ile Tyr Pro Phe Phe Leu Gly Gly Leu
580 585 590

Ala Arg Thr Ser Gly Trp Thr Val Asn His Ser Arg Ile Ile Ala Phe
595 600 605

```
Ser Leu Asp Gly Lys Ser Gly Pro Leu Pro Lys Gln Asn Asp Gln Gly
610                 615                 620

Phe Leu Pro Val Lys Pro Pro Ala Gln Phe Asp Ser Lys Arg Thr Asp
625                 630                 635                 640

Asn Gly Tyr Phe Gln Phe Gln Thr Tyr Cys Ala Ala Cys His Gly Asp
                645                 650                 655

Asn Ala Glu Gly Ala Gly Val Leu Pro Asp Leu Arg Trp Ser Gly Ser
            660                 665                 670

Ile Arg His Glu Asp Ala Phe Tyr Asn Val Val Gly Arg Gly Ala Leu
        675                 680                 685

Thr Ala Tyr Gly Met Asp Arg Leu His Gly Asn Met Asn Pro Thr Glu
    690                 695                 700

Ile Glu Asp Ile Arg Gln Phe Leu Ile Lys Arg Ala Asn Glu Thr Tyr
705                 710                 715                 720

Gln Arg Glu Val Asp Ala Arg Lys Asn Ala Asp Gly Ile Pro Glu Gln
                725                 730                 735

Leu Pro
738
```

4. SEQ ID NO: 4:

SEQUENCE TYPE:	amino acid
SEQUENCE LENGTH:	468
MOLECULE TYPE:	protein
ORIGINAL SOURCE ORGANISM:	Acetobacter altoacetigenes MH-24

```
Met Ile Asn Arg Leu Lys Val Thr Phe Ser Ala Ala Ala Phe Ser Leu
 1               5                  10                  15

Leu Ala Gly Thr Ala Leu Ala Gln Thr Pro Asp Ala Asp Ser Ala Leu
            20                  25                  30

Val Gln Lys Gly Ala Tyr Val Ala Arg Leu Gly Asp Cys Val Ala Cys
        35                  40                  45

His Thr Ala Leu His Gly Gln Ser Tyr Ala Gly Gly Leu Glu Ile Lys
    50                  55                  60

Ser Pro Ile Gly Thr Ile Tyr Ser Thr Asn Ile Thr Pro Asp Pro Thr
65                  70                  75                  80

Tyr Gly Ile Gly Arg Tyr Thr Phe Ala Glu Phe Asp Glu Ala Val Arg
                85                  90                  95

His Gly Ile Arg Lys Asp Gly Ser Thr Leu Tyr Pro Ala Met Pro Tyr
            100                 105                 110

Pro Ser Phe Ser Arg Met Thr Lys Glu Asp Met Gln Ala Leu Tyr Ala
        115                 120                 125

Tyr Phe Met His Gly Val Lys Pro Val Ala Gln Pro Asp Lys Gln Pro
    130                 135                 140

Asp Ile Ser Trp Pro Leu Ser Met Arg Trp Pro Leu Gly Ile Trp Arg
145                 150                 155                 160

Met Met Phe Ser Pro Ser Pro Lys Asp Phe Thr Pro Ala Pro Gly Thr
                165                 170                 175

Asp Pro Glu Ile Ala Arg Gly Asp Tyr Leu Val Thr Gly Pro Gly His
            180                 185                 190

Cys Gly Ala Cys His Thr Pro Arg Gly Phe Ala Met Gln Glu Lys Ala
        195                 200                 205

Leu Asp Ala Ala Gly Gly Pro Asp Phe Leu Ser Gly Gly Ala Pro Ile
    210                 215                 220

Asp Asn Trp Val Ala Pro Ser Leu Arg Asn Asp Pro Val Val Gly Leu
225                 230                 235                 240

Gly Arg Trp Ser Glu Asp Asp Ile Tyr Thr Phe Leu Lys Ser Gly Arg
                245                 250                 255

Ile Asp His Ser Ala Val Phe Gly Gly Met Gly Asp Val Val Ala Trp
            260                 265                 270

Ser Thr Gln Tyr Phe Thr Asp Asp Asp Leu His Ala Ile Ala Lys Tyr
        275                 280                 285

Leu Lys Ser Leu Pro Pro Val Pro Pro Ser Gln Gly Asn Tyr Thr Tyr
    290                 295                 300
```

```
Asp Pro Ser Thr Ala Asn Met Leu Ala Ser Gly Asn Thr Ala Ser Val
305                 310                 315                 320

Pro Gly Ala Asp Thr Tyr Val Lys Glu Cys Ala Ile Cys His Arg Asn
                325                 330                 335

Asp Gly Gly Gly Val Ala Arg Met Phe Pro Pro Leu Ala Gly Asn Pro
            340                 345                 350

Val Val Val Thr Glu Asn Pro Thr Ser Leu Val Asn Val Ile Ala His
        355                 360                 365

Gly Gly Val Leu Pro Pro Ser Asn Trp Ala Pro Ser Ala Val Ala Met
    370                 375                 380

Pro Gly Tyr Ser Lys Ser Leu Ser Ala Gln Gln Ile Ala Asp Val Val
385                 390                 395                 400

Asn Phe Ile Arg Thr Ser Trp Gly Asn Lys Ala Pro Gly Thr Val Thr
                405                 410                 415

Ala Ala Asp Val Thr Lys Leu Arg Asp Thr Gly Ala Pro Val Ser Ser
            420                 425                 430

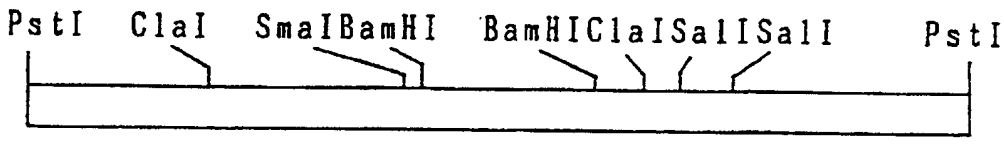
Ser Gly Trp Asn Ser Val Ser Ser Gly Trp Ser Val Phe Leu Pro Gln
        435                 440                 445

Pro Tyr Gly Ser Gly Trp Thr Phe Ala Pro Gln Thr His Thr Gly Gln
    450                 455                 460

Asp Ala Ala Gln
465         468
```

Claims

1. A structural gene of the membrane-bound alcohol dehydrogenase complex having a size of about 7.0 kilo base being derived from a microorganism belonging to the genus Acetobacter and having the following restriction enzyme map:



2. The structural gene according to claim 1, wherein said membrane-bound alcohol dehydrogenase complex is composed of proteins having a molecular weight of about 72,000 and a molecular weight of about 44,000.

3. The structural gene according to claim 2, encoding a protein with a molecular weight of about 72,000, and having the following nucleotide sequence:

```
        10         20         30         40         50
ATGATTTCTGCCGTTTTCGGAAAAAGACGTTCTCTGAGCAGAACGCTTACAGCCGGAACG

        70         80         90        100        110
ATATGTGCGGCTCTCATCTCCGGGTATGCCACCATGGCATCCGCAGATGACGGGCAGGGC

       130        140        150        160        170
GCCACGGGGGAAGCGATCATCCATGCCGATGATCACCCCGGTAACTGGATGACCTATGGC

       190        200        210        220        230
CGCACCTATTCTGACCAGCGCTACAGCCCGCTGGATCAGATCAACCGTTCCAATGTCGGT

       250        260        270        280        290
AACCTGAAGCTGGCCTGGTATCTGGACCTTGATACCAACCGTGGCCAGGAAGGCACGCCC

       310        320        330        340        350
CTGGTTATTGATGGCGTCATGTACGCCACCACCAACTGGAGCATGATGAAAGCCGTCGAC

       370        380        390        400        410
GCCGCAACCGGCAAGCTGCTGTGGTCCTATGACCCGCGCGTGCCCGGCAACATTGCCGAC

       430        440        450        460        470
AAGGGCTGCTGTGACACGGTCAACCGTGGCGCGGCATACTGGAATGGCAAGGTCTATTTC

       490        500        510        520        530
GGCACGTTCGACGGTCGCCTGATCGCGCTGGACGCCAAGACCGGCAAGCTGGTCTGGAGC

       550        560        570        580        590
GTCAACACCATTCCGCCCGAAGCGGAACTGGGCAAGCAGCGTTCCTATACGGTTGACGGC

       610        620        630        640        650
GCGCCCCGTATCGCCAAGGGCCGCGTGATCATCGGTAACGGTGGTTCCGAATTCGGTGCC

       670        680        690        700        710
CGTGGCTTCGTCAGCGCGTTCGATGCGGAAACCGGCAAGGTCGACTGGCGCTTCTTCACG

       730        740        750        760        770
GTTCCGAACCCCAAGAACGAACCGGACGCTGCATCCGACAGCGTGCTGATGAACAAGGCC

       790        800        810        820        830
TACCAGACCTGGAGCCCGACCGGCGCCTGGACCCGCCAGGGTGGCGGCGGCACGGTATGG

       850        860        870        880        890
GATTCCATCGTGTATGACCCCGTGGCCGACCTGGTCTACCTGGGCGTTGGCAACGGTTCG

       910        920        930        940        950
CCGTGGAACTACAAGTACCGTTCCGAAGGCAAGGGCGACAACCTGTTCCTGGGCAGCATC
```

970 980 990 1000 1010
GTCGCACTGAAGCCGGAAACCGGCGAATACGTCTGGCATTTCCAGGAAACGCCGATGGAC

1030 1040 1050 1060 1070
CAGTGGGACTTCACCTCGGACCAGCAGATCATGACGCTTGACCTGCCGATCAATGGTGAA

1090 1100 1110 1120 1130
ACCCGCCACGTCATCGTCCATGCGCGCAAGAACGGCTTCTTCTACATCATCGATGCGAAG

1150 1160 1170 1180 1190
ACCGGTGAGTTCATCTCGGGCAAGAACTACGTCTATGTGAACTGGGCCAGCGGCCTTGAT

1210 1220 1230 1240 1250
CCCAAGACCGGCCGTCCGATCTACAACCCCGATGCGCTCTACACCCTTACGGGCAAGGAA

1270 1280 1290 1300 1310
TGGTACGGCATTCCGGGTGACCTTGGCGGCCATAACTTCGCGGCCATGGCGTTCAGCCCC

1330 1340 1350 1360 1370
AAGACCGGGCTGGTCTATATTCCGGCGCAGCAGGTTCCGTTCCTGTACACCAATCAGGTC

1390 1400 1410 1420 1430
GGTGGCTTCACGCCGCACCCCGACAGCTGGAACCTGGGTCTGGACATGAACAAGGTCGGT

1450 1460 1470 1480 1490
ATTCCCGACTCGCCTGAAGCCAAGCAGGCCTTCGTGAAGGACCTGAAGGGCTGGATCGTG

1510 1520 1530 1540 1550
GCCTGGGATCCGCAGAAGCAGGCTGAAGCATGGCGCGTGGACCACAAGGGGCCGTGGAAC

1570 1580 1590 1600 1610
GGCGGTATCCTGGCAACTGGCGGCGACCTGCTGTTCCAGGGCTTGGCGAACGGCGAATTC

1630 1640 1650 1660 1670
CATGCCTATGACGCGACGAACGGTTCCGACCTGTTCCACTTCGCGGCGGACAGCGGCATC

1690 1700 1710 1720 1730
ATCGCACCGCCTGTGACCTACCTTGCCAATGGCAAGCAGTATGTTGCGGTTGAAGTGGGC

1750 1760 1770 1780 1790
TGGGGCGGCATCTATCCGTTCTTCCTTGGTGGCCTGGCCCGTACCAGCGGCTGGACCGTC

1810 1820 1830 1840 1850
AACCACTCGCGCATCATTGCCTTCTCGCTCGATGGCAAGTCCGGCCCGCTGCCCAAGCAG

1870 1880 1890 1900 1910
AATGACCAGGGCTTCCTGCCCGTCAAGCCGCCGGCACAGTTCGACAGCAAGCGTACCGAT

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      1930       1940       1950       1960       1970
AACGGTTACTTCCAGTTCCAGACCTATTGCGCCGCCTGTCATGGCGATAACGCAGAAGGT

      1990       2000       2010       2020       2030
GCCGGTGTGCTGCCTGACCTGCGCTGGTCCGGGTCCATCCGTCATGAGGACGCGTTCTAC

      2050       2060       2070       2080       2090
AATGTTGTCGGCCGCGGCGCGCTTACCGCCTACGGTATGGATCGCTTGCACGGTAACATG

      2110       2120       2130       2140       2150
AACCCGACCGAGATTGAGGACATCCGCCAGTTCCTGATCAAGCGTGCGAACGAGACCTAT

      2170       2180       2190       2200       2210
CAGAGGGAAGTTGATGCCCGGAAGAACGCTGACGGTATCCCCGAGCAGCTGCCG
```

4. The structural gene according to claim 2, encoding a protein with a molecular weight of about 44,000, and having the following nucleotide sequence:

```
        10         20         30         40         50
ATGATCAACAGACTTAAGGTGACATTCAGCGCGGCAGCGTTTAGTCTGCTGGCAGGGACG
        70         80         90        100        110
GCATTGGCACAGACGCCAGATGCTGACTCCGCGCTGGTCCAGAAGGGGGCATATGTCGCG
       130        140        150        160        170
CGACTGGGTGACTGCGTAGCATGTCATACCGCTCTCCATGGACAGTCGTACGCAGGCGGG
       190        200        210        220        230
CTTGAAATCAAGAGCCCGATCGGTACGATCTACTCCACGAACATCACACCGGACCCGACC
       250        260        270        280        290
TACGGTATCGGTCGCTACACCTTCGCCGAATTCGACGAAGCCGTGCGCCATGGTATCCGC
       310        320        330        340        350
AAGGACGGTTCCACGCTGTATCCGGCCATGCCGTATCCCTCCTTCTCGCGCATGACGAAG
       370        380        390        400        410
GAAGACATGCAGGCGCTGTATGCGTACTTCATGCATGGGGTGAAGCCGGTCGCGCAGCCG
       430        440        450        460        470
GACAAGCAGCCGGACATCTCCTGGCCCTTGTCCATGCGCTGGCCGCTGGGCATCTGGCGC
       490        500        510        520        530
ATGATGTTCTCGCCTTCGCCGAAGGACTTCACGCCGGCGCCAGGCACGGATCCTGAAATC
       550        560        570        580        590
GCACGTGGCGATTATCTGGTTACCGGCCCCGGGCATTGCGGTGCGTGTCATACCCCGCGT
       610        620        630        640        650
GGCTTCGCCATGCAGGAAAAGGCGCTGGACGCTGCCGGTGGTCCTGACTTCCTGTCCGGT
       670        680        690        700        710
GGCGCACCGATCGACAACTGGGTCGCGCCGAGCCTGCGCAACGATCCTGTCGTTGGTCTG
       730        740        750        760        770
GGCCGCTGGTCCGAGGATGACATCTACACCTTCCTGAAGTCCGGCCGTATCGACCACTCC
       790        800        810        820        830
GCCGTGTTCGGTGGCATGGGCGATGTGGTGGCATGGAGCACCCAGTACTTCACCGATGAC
       850        860        870        880        890
GACCTGCACGCCATCGCGAAGTACCTGAAGAGCCTGCCGCCGGTGCCGCCGTCACAGGGC
       910        920        930        940        950
AACTACACCTACGATCCGTCCACCGCGAACATGCTGGCTTCGGGTAATACCGCCAGCGTT
```

970 980 990 1000 1010

CCGGGTGCTGATACGTATGTGAAGGAATGCGCCATCTGTCACCGTAACGACGGTGGTGGC

1030 1040 1050 1060 1070

GTGGCCCGCATGTTCCCGCCGCTGGCTGGCAACCCGGTTGTCGTGACCGAGAACCCGACC

1090 1100 1110 1120 1130

TCGCTGGTGAACGTGATTGCGCATGGTGGCGTGCTGCCGCCGAGCAACTGGGCACCGTCC

1150 1160 1170 1180 1190

GCAGTGGCAATGCCGGGTTACAGCAAGTCGCTGTCCGCCCAGCAGATTGCTGATGTGGTC

1210 1220 1230 1240 1250

AACTTCATCCGCACCAGCTGGGGCAACAAGGCGCCCGGCACCGTTACGGCTGCGGATGTT

1270 1280 1290 1300 1310

ACCAAGCTGCGCGACACGGGCGCCCCGGTTTCCAGCTCTGGCTGGAACAGCGTGAGCAGC

1330 1340 1350 1360 1370

GGCTGGTCGGTCTTCCTGCCGCAGCCTTACGGCTCGGGCTGGACGTTTGCCCCGCAGACG

1390 1400

CACACCGGTCAGGACGCCGCACAG

5. The structural gene according to claim 2, encoding a protein with a molecular weight of about 72,000 and the following amino acid sequence:

10 20
MetIleSerAlaValPheGlyLysArgArgSerLeuSerArgThrLeuThrAlaGlyThr
30 40
IleCysAlaAlaLeuIleSerGlyTyrAlaThrMetAlaSerAlaAspAspGlyGlnGly
50 60
AlaThrGlyGluAlaIleIleHisAlaAspAspHisProGlyAsnTrpMetThrTyrGly
70 80
ArgThrTyrSerAspGlnArgTyrSerProLeuAspGlnIleAsnArgSerAsnValGly
90 100
AsnLeuLysLeuAlaTrpTyrLeuAspLeuAspThrAsnArgGlyGlnGluGlyThrPro
110 120
LeuValIleAspGlyValMetTyrAlaThrThrAsnTrpSerMetMetLysAlaValAsp
130 140
AlaAlaThrGlyLysLeuLeuTrpSerTyrAspProArgValProGlyAsnIleAlaAsp
150 160
LysGlyCysCysAspThrValAsnArgGlyAlaAlaTyrTrpAsnGlyLysValTyrPhe
170 180
GlyThrPheAspGlyArgLeuIleAlaLeuAspAlaLysThrGlyLysLeuValTrpSer
190 200
ValAsnThrIleProProGluAlaGluLeuGlyLysGlnArgSerTyrThrValAspGly
210 220
AlaProArgIleAlaLysGlyArgValIleIleGlyAsnGlyGlySerGluPheGlyAla
230 240
ArgGlyPheValSerAlaPheAspAlaGluThrGlyLysValAspTrpArgPhePheThr
250 260
ValProAsnProLysAsnGluProAspAlaAlaSerAspSerValLeuMetAsnLysAla
270 280
TyrGlnThrTrpSerProThrGlyAlaTrpThrArgGlnGlyGlyGlyGlyThrValTrp
290 300
AspSerIleValTyrAspProValAlaAspLeuValTyrLeuGlyValGlyAsnGlySer
310 320
ProTrpAsnTyrLysTyrArgSerGluGlyLysGlyAspAsnLeuPheLeuGlySerIle
330 340
ValAlaLeuLysProGluThrGlyGluTyrValTrpHisPheGlnGluThrProMetAsp
350 360
GlnTrpAspPheThrSerAspGlnGlnIleMetThrLeuAspLeuProIleAsnGlyGlu
370 380
ThrArgHisValIleValHisAlaArgLysAsnGlyPhePheTyrIleIleAspAlaLys
390 400
ThrGlyGluPheIleSerGlyLysAsnTyrValTyrValAsnTrpAlaSerGlyLeuAsp

410 420
ProLysThrGlyArgProIleTyrAsnProAspAlaLeuTyrThrLeuThrGlyLysGlu
430 440
TrpTyrGlyIleProGlyAspLeuGlyGlyHisAsnPheAlaAlaMetAlaPheSerPro
450 460
LysThrGlyLeuValTyrIleProAlaGlnGlnValProPheLeuTyrThrAsnGlnVal
470 480
GlyGlyPheThrProHisProAspSerTrpAsnLeuGlyLeuAspMetAsnLysValGly
490 500
IleProAspSerProGluAlaLysGlnAlaPheValLysAspLeuLysGlyTrpIleVal
510 520
AlaTrpAspProGlnLysGlnAlaGluAlaTrpArgValAspHisLysGlyProTrpAsn
530 540
GlyGlyIleLeuAlaThrGlyGlyAspLeuLeuPheGlnGlyLeuAlaAsnGlyGluPhe
550 560
HisAlaTyrAspAlaThrAsnGlySerAspLeuPheHisPheAlaAlaAspSerGlyIle
570 580
IleAlaProProValThrTyrLeuAlaAsnGlyLysGlnTyrValAlaValGluValGly
590 600
TrpGlyGlyIleTyrProPhePheLeuGlyGlyLeuAlaArgThrSerGlyTrpThrVal
610 620
AsnHisSerArgIleIleAlaPheSerLeuAspGlyLysSerGlyProLeuProLysGln
630 640
AsnAspGlnGlyPheLeuProValLysProProAlaGlnPheAspSerLysArgThrAsp
650 660
AsnGlyTyrPheGlnPheGlnThrTyrCysAlaAlaCysHisGlyAspAsnAlaGluGly
670 680
AlaGlyValLeuProAspLeuArgTrpSerGlySerIleArgHisGluAspAlaPheTyr
690 700
AsnValValGlyArgGlyAlaLeuThrAlaTyrGlyMetAspArgLeuHisGlyAsnMet
710 720
AsnProThrGluIleGluAspIleArgGlnPheLeuIleLysArgAlaAsnGluThrTyr
730
GlnArgGluValAspAlaArgLysAsnAlaAspGlyIleProGluGlnLeuPro

6. The structural gene according to claim 2, encoding a protein having a molecular weight of about 44,000 and the following amino acid sequence:

```
                                                  10                  20
MetIleAsnArgLeuLysValThrPheSerAlaAlaAlaPheSerLeuLeuAlaGlyThr
                                                  30                  40
AlaLeuAlaGlnThrProAspAlaAspSerAlaLeuValGlnLysGlyAlaTyrValAla
                                                  50                  60
ArgLeuGlyAspCysValAlaCysHisThrAlaLeuHisGlyGlnSerTyrAlaGlyGly
                                                  70                  80
LeuGluIleLysSerProIleGlyThrIleTyrSerThrAsnIleThrProAspProThr
                                                  90                  100
TyrGlyIleGlyArgTyrThrPheAlaGluPheAspGluAlaValArgHisGlyIleArg
                                                  110                 120
LysAspGlySerThrLeuTyrProAlaMetProTyrProSerPheSerArgMetThrLys
                                                  130                 140
GluAspMetGlnAlaLeuTyrAlaTyrPheMetHisGlyValLysProValAlaGlnPro
                                                  150                 160
AspLysGlnProAspIleSerTrpProLeuSerMetArgTrpProLeuGlyIleTrpArg
                                                  170                 180
MetMetPheSerProSerProLysAspPheThrProAlaProGlyThrAspProGluIle
                                                  190                 200
AlaArgGlyAspTyrLeuValThrGlyProGlyHisCysGlyAlaCysHisThrProArg
```

```
                                    210                                 220
GlyPheAlaMetGlnGluLysAlaLeuAspAlaAlaGlyGlyProAspPheLeuSerGly
                                    230                                 240
GlyAlaProIleAspAsnTrpValAlaProSerLeuArgAsnAspProValValGlyLeu
                                    250                                 260
GlyArgTrpSerGluAspAspIleTyrThrPheLeuLysSerGlyArgIleAspHisSer
                                    270                                 280
AlaValPheGlyGlyMetGlyAspValValAlaTrpSerThrGlnTyrPheThrAspAsp
                                    290                                 300
AspLeuHisAlaIleAlaLysTyrLeuLysSerLeuProProValProProSerGlnGly
                                    310                                 320
AsnTyrThrTyrAspProSerThrAlaAsnMetLeuAlaSerGlyAsnThrAlaSerVal
                                    330                                 340
ProGlyAlaAspThrTyrValLysGluCysAlaIleCysHisArgAsnAspGlyGlyGly
                                    350                                 360
ValAlaArgMetPheProProLeuAlaGlyAsnProValValValThrGluAsnProThr
                                    370                                 380
SerLeuValAsnValIleAlaHisGlyGlyValLeuProProSerAsnTrpAlaProSer
                                    390                                 400
AlaValAlaMetProGlyTyrSerLysSerLeuSerAlaGlnGlnIleAlaAspValVal

                                    410                                 420
AsnPheIleArgThrSerTrpGlyAsnLysAlaProGlyThrValThrAlaAlaAspVal
                                    430                                 440
ThrLysLeuArgAspThrGlyAlaProValSerSerSerGlyTrpAsnSerValSerSer
                                    450                                 460
GlyTrpSerValPheLeuProGlnProTyrGlySerGlyTrpThrPheAlaProGlnThr

HisThrGlyGlnAspAlaAlaGln
```

7. A plasmid containing a structural gene according to any one of claims 1 to 6.

8. An acetic acid bacterium belonging to the genus Acetobacter or the genus Gluconobacter transformed with a plasmid according to claim 7.

9. A process for the preparation of a membrane-bound alcohol-dehydrogenase complex wherein an acetic acid bacterium according to claim 8 is cultivated under suitable conditions.

10. The process according to claim 9, additionally comprising the step of isolating said alcohol-dehydrogenase complex.

FIG. 1

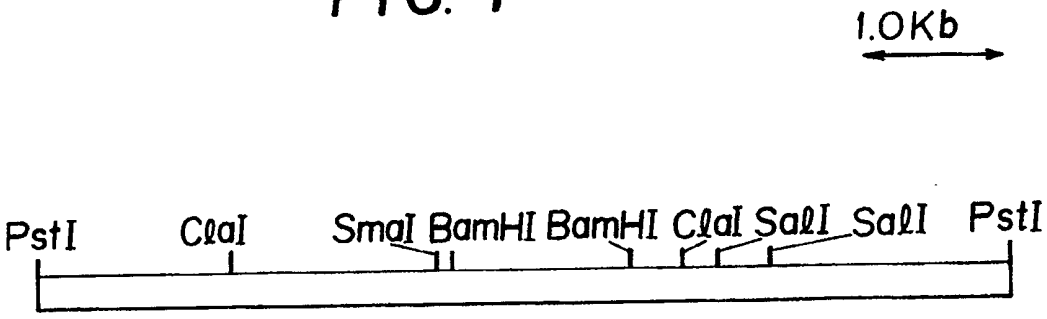
1.0Kb
PstI
ClaI
SmaI
BamHI
BamHI
ClaI
SalI
SalI
PstI

FIG. 2

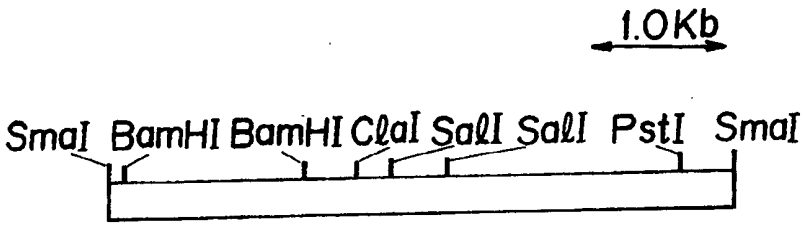
1.0Kb
SmaI
BamHI
BamHI
ClaI
SalI
SalI
PstI
SmaI

FIG. 3-1

```
        10         20         30         40         50
ATGATTTCTGCCGTTTTCGGAAAAAGACGTTCTCTGAGCAGAACGCTTACAGCCGGAACG
MetIleSerAlaValPheGlyLysArgArgSerLeuSerArgThrLeuThrAlaGlyThr
        70         80         90        100        110
ATATGTGCGGCTCTCATCTCCGGGTATGCCACCATGGCATCCGCAGATGACGGGCAGGGC
IleCysAlaAlaLeuIleSerGlyTyrAlaThrMetAlaSerAlaAspAspGlyGlnGly
       130        140        150        160        170
GCCACGGGGGAAGCGATCATCCATGCCGATGATCACCCCGGTAACTGGATGACCTATGGC
AlaThrGlyGluAlaIleIleHisAlaAspAspHisProGlyAsnTrpMetThrTyrGly
       190        200        210        220        230
CGCACCTATTCTGACCAGCGCTACAGCCCGCTGGATCAGATCAACCGTTCCAATGTCGGT
ArgThrTyrSerAspGlnArgTyrSerProLeuAspGlnIleAsnArgSerAsnValGly
       250        260        270        280        290
AACCTGAAGCTGGCCTGGTATCTGGACCTTGATACCAACCGTGGCCAGGAAGGCACGCCC
AsnLeuLysLeuAlaTrpTyrLeuAspLeuAspThrAsnArgGlyGlnGluGlyThrPro
       310        320        330        340        350
CTGGTTATTGATGGCGTCATGTACGCCACCACCAACTGGAGCATGATGAAAGCCGTCGAC
LeuValIleAspGlyValMetTyrAlaThrThrAsnTrpSerMetMetLysAlaValAsp
       370        380        390        400        410
GCCGCAACCGGCAAGCTGCTGTGGTCCTATGACCCGCGCGTGCCCGGCAACATTGCCGAC
AlaAlaThrGlyLysLeuLeuTrpSerTyrAspProArgValProGlyAsnIleAlaAsp
       430        440        450        460        470
AAGGGCTGCTGTGACACGGTCAACCGTGGCGCGGCATACTGGAATGGCAAGGTCTATTTC
LysGlyCysCysAspThrValAsnArgGlyAlaAlaTyrTrpAsnGlyLysValTyrPhe
       490        500        510        520        530
GGCACGTTCGACGGTCGCCTGATCGCGCTGGACGCCAAGACCGGCAAGCTGGTCTGGAGC
GlyThrPheAspGlyArgLeuIleAlaLeuAspAlaLysThrGlyLysLeuValTrpSer
       550        560        570        580        590
GTCAACACCATTCCGCCCGAAGCGGAACTGGGCAAGCAGCGTTCCTATACGGTTGACGGC
ValAsnThrIleProProGluAlaGluLeuGlyLysGlnArgSerTyrThrValAspGly
```

FIG. 3-2

```
      610        620        630        640        650
GCGCCCCGTATCGCCAAGGGCCGCGTGATCATCGGTAACGGTGGTTCCGAATTCGGTGCC
AlaProArgIleAlaLysGlyArgValIleIleGlyAsnGlyGlySerGluPheGlyAla
      670        680        690        700        710
CGTGGCTTCGTCAGCGCGTTCGATGCGGAAACCGGCAAGGTCGACTGGCGCTTCTTCACG
ArgGlyPheValSerAlaPheAspAlaGluThrGlyLysValAspTrpArgPhePheThr
      730        740        750        760        770
GTTCCGAACCCCAAGAACGAACCGGACGCTGCATCCGACAGCGTGCTGATGAACAAGGCC
ValProAsnProLysAsnGluProAspAlaAlaSerAspSerValLeuMetAsnLysAla
      790        800        810        820        830
TACCAGACCTGGAGCCCGACCGGCGCCTGGACCCGCCAGGGTGGCGGCGGCACGGTATGG
TyrGlnThrTrpSerProThrGlyAlaTrpThrArgGlnGlyGlyGlyGlyThrValTrp
      850        860        870        880        890
GATTCCATCGTGTATGACCCCGTGGCCGACCTGGTCTACCTGGGCGTTGGCAACGGTTCG
AspSerIleValTyrAspProValAlaAspLeuValTyrLeuGlyValGlyAsnGlySer
      910        920        930        940        950
CCGTGGAACTACAAGTACCGTTCCGAAGGCAAGGGCGACAACCTGTTCCTGGGCAGCATC
ProTrpAsnTyrLysTyrArgSerGluGlyLysGlyAspAsnLeuPheLeuGlySerIle
      970        980        990       1000       1010
GTCGCACTGAAGCCGGAAACCGGCGAATACGTCTGGCATTTCCAGGAAACGCCGATGGAC
ValAlaLeuLysProGluThrGlyGluTyrValTrpHisPheGlnGluThrProMetAsp
     1030       1040       1050       1060       1070
CAGTGGGACTTCACCTCGGACCAGCAGATCATGACGCTTGACCTGCCGATCAATGGTGAA
GlnTrpAspPheThrSerAspGlnGlnIleMetThrLeuAspLeuProIleAsnGlyGlu
     1090       1100       1110       1120       1130
ACCCGCCACGTCATCGTCCATGCGCGCAAGAACGGCTTCTTCTACATCATCGATGCGAAG
ThrArgHisValIleValHisAlaArgLysAsnGlyPhePheTyrIleIleAspAlaLys
     1150       1160       1170       1180       1190
ACCGGTGAGTTCATCTCGGGCAAGAACTACGTCTATGTGAACTGGGCCAGCGGCCTTGAT
ThrGlyGluPheIleSerGlyLysAsnTyrValTyrValAsnTrpAlaSerGlyLeuAsp
```

FIG. 3-3

```
      1210       1220       1230       1240       1250
CCCAAGACCGGCCGTCCGATCTACAACCCCGATGCGCTCTACACCCTTACGGGCAAGGAA
ProLysThrGlyArgProIleTyrAsnProAspAlaLeuTyrThrLeuThrGlyLysGlu
      1270       1280       1290       1300       1310
TGGTACGGCATTCCGGGTGACCTTGGCGGCCATAACTTCGCGGCCATGGCGTTCAGCCCC
TrpTyrGlyIleProGlyAspLeuGlyGlyHisAsnPheAlaAlaMetAlaPheSerPro
      1330       1340       1350       1360       1370
AAGACCGGGCTGGTCTATATTCCGGCGCAGCAGGTTCCGTTCCTGTACACCAATCAGGTC
LysThrGlyLeuValTyrIleProAlaGlnGlnValProPheLeuTyrThrAsnGlnVal
      1390       1400       1410       1420       1430
GGTGGCTTCACGCCGCACCCCGACAGCTGGAACCTGGGTCTGGACATGAACAAGGTCGGT
GlyGlyPheThrProHisProAspSerTrpAsnLeuGlyLeuAspMetAsnLysValGly
      1450       1460       1470       1480       1490
ATTCCCGACTCGCCTGAAGCCAAGCAGGCCTTCGTGAAGGACCTGAAGGGCTGGATCGTG
IleProAspSerProGluAlaLysGlnAlaPheValLysAspLeuLysGlyTrpIleVal
      1510       1520       1530       1540       1550
GCCTGGGATCCGCAGAAGCAGGCTGAAGCATGGCGCGTGGACCACAAGGGGCCGTGGAAC
AlaTrpAspProGlnLysGlnAlaGluAlaTrpArgValAspHisLysGlyProTrpAsn
      1570       1580       1590       1600       1610
GGCGGTATCCTGGCAACTGGCGGCGACCTGCTGTTCCAGGGCTTGGCGAACGGCGAATTC
GlyGlyIleLeuAlaThrGlyGlyAspLeuLeuPheGlnGlyLeuAlaAsnGlyGluPhe
      1630       1640       1650       1660       1670
CATGCCTATGACGCGACGAACGGTTCCGACCTGTTCCACTTCGCGGCGGACAGCGGCATC
HisAlaTyrAspAlaThrAsnGlySerAspLeuPheHisPheAlaAlaAspSerGlyIle
      1690       1700       1710       1720       1730
ATCGCACCGCCTGTGACCTACCTTGCCAATGGCAAGCAGTATGTTGCGGTTGAAGTGGGC
IleAlaProProValThrTyrLeuAlaAsnGlyLysGlnTyrValAlaValGluValGly
      1750       1760       1770       1780       1790
TGGGGCGGCATCTATCCGTTCTTCCTTGGTGGCCTGGCCCGTACCAGCGGCTGGACCGTC
TrpGlyGlyIleTyrProPhePheLeuGlyGlyLeuAlaArgThrSerGlyTrpThrVal
```

FIG.3-4

```
      1810       1820       1830       1840       1850
AACCACTCGCGCATCATTGCCTTCTCGCTCGATGGCAAGTCCGGCCCGCTGCCCAAGCAG
AsnHisSerArgIleIleAlaPheSerLeuAspGlyLysSerGlyProLeuProLysGln
      1870       1880       1890       1900       1910
AATGACCAGGGCTTCCTGCCCGTCAAGCCGCCGGCACAGTTCGACAGCAAGCGTACCGAT
AsnAspGlnGlyPheLeuProValLysProProAlaGlnPheAspSerLysArgThrAsp
      1930       1940       1950       1960       1970
AACGGTTACTTCCAGTTCCAGACCTATTGCGCCGCCTGTCATGGCGATAACGCAGAAGGT
AsnGlyTyrPheGlnPheGlnThrTyrCysAlaAlaCysHisGlyAspAsnAlaGluGly
      1990       2000       2010       2020       2030
GCCGGTGTGCTGCCTGACCTGCGCTGGTCCGGGTCCATCCGTCATGAGGACGCGTTCTAC
AlaGlyValLeuProAspLeuArgTrpSerGlySerIleArgHisGluAspAlaPheTyr
      2050       2060       2070       2080       2090
AATGTTGTCGGCCGCGGCGCGCTTACCGCCTACGGTATGGATCGCTTGCACGGTAACATG
AsnValValGlyArgGlyAlaLeuThrAlaTyrGlyMetAspArgLeuHisGlyAsnMet
      2110       2120       2130       2140       2150
AACCCGACCGAGATTGAGGACATCCGCCAGTTCCTGATCAAGCGTGCGAACGAGACCTAT
AsnProThrGluIleGluAspIleArgGlnPheLeuIleLysArgAlaAsnGluThrTyr
      2170       2180       2190       2200       2210
CAGAGGGAAGTTGATGCCCGGAAGAACGCTGACGGTATCCCCGAGCAGCTGCCG
GlnArgGluValAspAlaArgLysAsnAlaAspGlyIleProGluGlnLeuPro
```

FIG. 4-1

```
        10         20         30         40         50
ATGATCAACAGACTTAAGGTGACATTCAGCGCGGCAGCGTTTAGTCTGCTGGCAGGGACG
MetIleAsnArgLeuLysValThrPheSerAlaAlaAlaPheSerLeuLeuAlaGlyThr
        70         80         90        100        110
GCATTGGCACAGACGCCAGATGCTGACTCCGCGCTGGTCCAGAAGGGGGCATATGTCGCG
AlaLeuAlaGlnThrProAspAlaAspSerAlaLeuValGlnLysGlyAlaTyrValAla
       130        140        150        160        170
CGACTGGGTGACTGCGTAGCATGTCATACCGCTCTCCATGGACAGTCGTACGCAGGCGGG
ArgLeuGlyAspCysValAlaCysHisThrAlaLeuHisGlyGlnSerTyrAlaGlyGly
       190        200        210        220        230
CTTGAAATCAAGAGCCCGATCGGTACGATCTACTCCACGAACATCACACCGGACCCGACC
LeuGluIleLysSerProIleGlyThrIleTyrSerThrAsnIleThrProAspProThr
       250        260        270        280        290
TACGGTATCGGTCGCTACACCTTCGCCGAATTCGACGAAGCCGTGCGCCATGGTATCCGC
TyrGlyIleGlyArgTyrThrPheAlaGluPheAspGluAlaValArgHisGlyIleArg
       310        320        330        340        350
AAGGACGGTTCCACGCTGTATCCGGCCATGCCGTATCCCTCCTTCTCGCGCATGACGAAG
LysAspGlySerThrLeuTyrProAlaMetProTyrProSerPheSerArgMetThrLys
       370        380        390        400        410
GAAGACATGCAGGCGCTGTATGCGTACTTCATGCATGGGGTGAAGCCGGTCGCGCAGCCG
GluAspMetGlnAlaLeuTyrAlaTyrPheMetHisGlyValLysProValAlaGlnPro
       430        440        450        460        470
GACAAGCAGCCGGACATCTCCTGGCCCTTGTCCATGCGCTGGCCGCTGGGCATCTGGCGC
AspLysGlnProAspIleSerTrpProLeuSerMetArgTrpProLeuGlyIleTrpArg
       490        500        510        520        530
ATGATGTTCTCGCCTTCGCCGAAGGACTTCACGCCGGCGCCAGGCACGGATCCTGAAATC
MetMetPheSerProSerProLysAspPheThrProAlaProGlyThrAspProGluIle
       550        560        570        580        590
GCACGTGGCGATTATCTGGTTACCGGCCCCGGGCATTGCGGTGCTGTCATACCCCGCGT
AlaArgGlyAspTyrLeuValThrGlyProGlyHisCysGlyAlaCysHisThrProArg
```

FIG. 4-2

```
        610         620         630         640         650
GGCTTCGCCATGCAGGAAAAGGCGCTGGACGCTGCCGGTGGTCCTGACTTCCTGTCCGGT
GlyPheAlaMetGlnGluLysAlaLeuAspAlaAlaGlyGlyProAspPheLeuSerGly
        670         680         690         700         710
GGCGCACCGATCGACAACTGGGTCGCGCCGAGCCTGCGCAACGATCCTGTCGTTGGTCTG
GlyAlaProIleAspAsnTrpValAlaProSerLeuArgAsnAspProValValGlyLeu
        730         740         750         760         770
GGCCGCTGGTCCGAGGATGACATCTACACCTTCCTGAAGTCCGGCCGTATCGACCACTCC
GlyArgTrpSerGluAspAspIleTyrThrPheLeuLysSerGlyArgIleAspHisSer
        790         800         810         820         830
GCCGTGTTCGGTGGCATGGGCGATGTGGTGGCATGGAGCACCCAGTACTTCACCGATGAC
AlaValPheGlyGlyMetGlyAspValValAlaTrpSerThrGlnTyrPheThrAspAsp
        850         860         870         880         890
GACCTGCACGCCATCGCGAAGTACCTGAAGAGCCTGCCGCCGGTGCCGCCGTCACAGGGC
AspLeuHisAlaIleAlaLysTyrLeuLysSerLeuProProValProProSerGlnGly
        910         920         930         940         950
AACTACACCTACGATCCGTCCACCGCGAACATGCTGGCTTCGGGTAATACCGCCAGCGTT
AsnTyrThrTyrAspProSerThrAlaAsnMetLeuAlaSerGlyAsnThrAlaSerVal
        970         980         990        1000        1010
CCGGGTGCTGATACGTATGTGAAGGAATGCGCCATCTGTCACCGTAACGACGGTGGTGGC
ProGlyAlaAspThrTyrValLysGluCysAlaIleCysHisArgAsnAspGlyGlyGly
       1030        1040        1050        1060        1070
GTGGCCCGCATGTTCCCGCCGCTGGCTGGCAACCCGGTTGTCGTGACCGAGAACCCGACC
ValAlaArgMetPheProProLeuAlaGlyAsnProValValValThrGluAsnProThr
       1090        1100        1110        1120        1130
TCGCTGGTGAACGTGATTGCGCATGGTGGCGTGCTGCCGCCGAGCAACTGGGCACCGTCC
SerLeuValAsnValIleAlaHisGlyGlyValLeuProProSerAsnTrpAlaProSer
       1150        1160        1170        1180        1190
GCAGTGGCAATGCCGGGTTACAGCAAGTCGCTGTCCGCCCAGCAGATTGCTGATGTGGTC
AlaValAlaMetProGlyTyrSerLysSerLeuSerAlaGlnGlnIleAlaAspValVal
```

FIG. 4-3

```
      1210       1220       1230       1240       1250
AACTTCATCCGCACCAGCTGGGGCAACAAGGCGCCCGGCACCGTTACGGCTGCGGATGTT
AsnPheIleArgThrSerTrpGlyAsnLysAlaProGlyThrValThrAlaAlaAspVal
      1270       1280       1290       1300       1310
ACCAAGCTGCGCGACACGGGCGCCCCGGTTTCCAGCTCTGGCTGGAACAGCGTGAGCAGC
ThrLysLeuArgAspThrGlyAlaProValSerSerSerGlyTrpAsnSerValSerSer
      1330       1340       1350       1360       1370
GGCTGGTCGGTCTTCCTGCCGCAGCCTTACGGCTCGGGCTGGACGTTTGCCCCGCAGACG
GlyTrpSerValPheLeuProGlnProTyrGlySerGlyTrpThrPheAlaProGlnThr
      1390       1400
CACACCGGTCAGGACGCCGCACAG
HisThrGlyGlnAspAlaAlaGln
```